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Assigned Report/Presentation #: TR-08-113
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Date: October 13, 2008

Submitting Author: David DeShazer, Ph.D.

Title: *Burkholderia mallei tssM* Encodes a Secreted Deubiquitinase That Is Expressed Inside Infected RAW 264.7 Murine Macrophages

USAMRIID Project/Plan Number: 06-4-2P-004 and Y1-AI-5004-01 (#22279)

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Is this an exact resubmission of a previously cleared work (yes/no)? No

If yes, indicate previous report/presentation # (skip rest of questions):

List all biological agent(s) described in this study: *Burkholderia mallei*

Are animals described in this study (yes/no)? Yes

If yes, indicate animal protocol #: AP-06-035 (and addendum dated 27 March 2007)

Are samples from humans or data from human patients described in this study (yes/no): No

If yes, indicate human protocol #:

Does this submission describe technology, materials, or information provided by another organization (yes/no)? Yes

If yes, indicate organization: Emory University, University of Georgia, Rocky Mountain Laboratories

Does USAMRIID have an agreement with this organization (yes/no)? No

Report Documentation Page

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Burkholderia mallei, a category B biothreat agent, is a facultative intracellular pathogen that causes the zoonotic disease glanders. The B. mallei VirAG two-component regulatory system activates the transcription of ~60 genes, including a large virulence gene cluster encoding a type VI secretion system (T6SS). The B. mallei tssM gene encodes a putative ubiquitin-specific protease that is physically linked to, and transcriptionally co-regulated with, the T6SS gene cluster. Mass spectrometry and immunoblot analysis demonstrated that TssM was secreted in a virAG-dependent manner in vitro. Surprisingly, the T6SS was found to be dispensable for the secretion of TssM. The C-terminal half of TssM, which contains Cys and His box motifs conserved in eukaryotic deubiquitinases, was purified and biochemically characterized. Recombinant TssM (rTssM) hydrolyzed multiple ubiquitinated substrates and the cysteine at position 102 was critical for enzymatic activity. The tssM gene was expressed within 1 h after uptake of B. mallei into RAW 264.7 murine macrophages, suggesting that the TssM deubiquitinase is produced in this intracellular niche. While the physiological substrate(s) is currently unknown, the TssM deubiquitinase may provide B. mallei a selective advantage in the intracellular environment during infection.

15. SUBJECT TERMS

Burkholderia mallei, ubiquitin, glanders, type VI secretion system, deubiquitinase, tssM

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Is the information described of potential commercial value (yes/no)? No

Submitting Author Acknowledgement (for USAMRIID-sponsored work):

I acknowledge that the information contained in this submission is technically accurate and that I have given proper consideration to authorship issues. In addition, I have deposited all relevant raw data, materials, strains, and reagents described in this submission in the appropriate institute archives.

David DeShazer Oct. 15, 2008

Name David DeShazer, Ph.D. Date

Co-Author Acknowledgement:

I am aware that I am listed as a co-author on the above-named submission, and I have reviewed the information and agree with its content. In addition, I consent to being listed as a co-author without prior notification if this submission is re-submitted to other forums in the future.

David M. Waag 16 Oct 2008

David M. Waag, Ph.D. Date

WJ Ribot 16 Oct 08

Wilson J. Ribot Date

16 Oct 08

Kevin Spurgers, Ph.D. Date

RG Panchal 22 Oct 08

Rekha G. Panchal, Ph.D. Date

Review:

Editorial:

The enclosed submission has been reviewed for technical, grammatical, and stylistic format. For resubmissions, content has been verified to be identical to that contained in the previous submission.

Katheryn Kenyon, M.S. Digitally signed by Katheryn Kenyon, M.S. DN: cn=Katheryn Kenyon, M.S., o=USAMRIID, ou=Editor, email=katheryn.kenyon@amedd.army.mil, c=US Date: 2008.10.14 09:38:31 -04'00'

Katheryn F. Kenyon Date

Animal Use:

The enclosed submission has been reviewed for compliance with the approved animal protocol indicated above by a Veterinary Medicine Division Lab Animal Veterinarian.

Carol Eisenhauer 29 Oct 08

Name Date

Human Use:

The enclosed submission has been reviewed for Human Use issues and has been determined to meet the following criteria:

( ) HUC approved.

( ) HUC Exemption Certificate on file.

N.A.

Name Date

Intellectual Property:

The enclosed submission has been reviewed for intellectual property and proprietary information restrictions and is recommended for release with the Distribution Statement indicated below.

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30 Oct 08

Name Date

Project Team Leader (for chartered project teams):

The enclosed submission has been reviewed for consistency with the objectives and impact on the project and I concur with its release.

Name Date

Division/Office Chief(s):

The enclosed publication or presentation has been reviewed for scientific quality of the personnel that I supervise and I concur with its release.

Name Date

DeShazer David

From: Gherardini, Frank (NIH/NIAID) [E] [FGherardini@niaid.nih.gov]
Sent: 09 October 2008 23:10
To: Deshazer, David Dr CIV USA USAMEDCOM
Subject: RE: Clearance

Dr. Deshazer, I acknowledge that I am a co-author on the manuscript and agree with the data presented.

Dr. Frank Gherardini

DeShazer David

From: jshanks@emory.edu
Sent: 24 September 2008 03:41
To: Deshazer, David Dr CIV USA USAMEDCOM
Cc: genekdw@emory.edu
Subject: Re: TssM manuscript

Hi David,

I'm on vacation this week, but checking my email occasionally. I just read your paper and found it very interesting: I really did not know much about TssM.

All discriptions and data for my work appear to be correct.

I enjoyed working in collaboration with you. Thank you for the honor of being placed as first author, but that is something you and Keith can discuss. It is clear that others have also invested much work into this research.

Let me know if I can be of further help.

Best regards,

John

DeShazer David

From: Paul Brett [brett_pj@yahoo.ca]
Sent: 07 October 2008 17:39
To: Dave DeShazer
Cc: Paul Brett
Subject: TssM manuscript

Hi Dave,

This is just a quick note to let you know that I have read and agree to the content of the manuscript titled " Burkholderia mallei tssM Encodes a Secreted Deubiquitinase That Is Expressed Inside Infected RAW 264.7 Cells".

Let me know if you need anything else.

Regards,

Paul

Paul J. Brett

DeShazer David

From: Mark Schell [schell@uga.edu]
Sent: 07 October 2008 14:09
To: Deshazer, David Dr CIV USA USAMEDCOM
Subject: Re: write up

Dear Dr. DeShazer:

I have read over the manuscript entitled "Burkholderia mallei tssM Encodes a Secreted Deubiquitinase That Is Expressed Inside Infected RAW 264.7 Cells" which will be submitted to Infection and Immunity. As a coauthor on this paper I am happy to inform you that I am satisfied and in agreement with its contents.

mas

--
Mark A. Schell
Dept. of Microbiology
Univ. of Georgia
Athens, GA 30602
PH: 706-542-2815
FAX 706-542-2674

DeShazer David

From: Keith Wilkinson [genekdw@emory.edu]
Sent: 08 October 2008 12:12
To: Deshazer, David Dr CIV USA USAMEDCOM
Subject: Authorship confimation

David

This note is to acknowledge that I have received a copy of the manuscript entitled "*Burkholderia mallei tssM* Encodes a Secreted Deubiquitinase That Is Expressed Inside Infected RAW 264.7 Cells" by John Shanks, Mary N. Burtnick, Paul J. Brett, David M. Waag, Kevin Spurgers, Wilson J. Ribot, Mark A. Schell, Rekha G. Panchal, Frank C. Gherardini, Keith D. Wilkinson, and David DeShazer

John Shanks and I approve of this submission and acknowledge that we will be listed as co-authors on this publication.

Keith

Keith D. Wilkinson, Ph.D.

Professor of Biochemistry

Director Graduate Division of Biological and Biomedical Sciences

Emory University

Atlanta, Georgia 30322

Biochemistry phone: (404) 727-5980 **GDBBS phone:** (404) 727-2545

E-Mail: Keith.Wilkinson@emory.edu

DeShazer David

From: Mary Burtnick [maryburtnick@yahoo.com]
Sent: 07 October 2008 17:34
To: David Dr CIV USA USAMEDCOM Deshazer
Subject: TssM manuscript

Dave,

I have read and agree to the content of your manuscript titled " *Burkholderia mallei tssM* Encodes a Secreted Deubiquitinase That Is Expressed Inside Infected RAW 264.7 Cells"

<!--[if !supportEmptyParas]--> <!--[endif]-->

If you need anything further please don't hesitate to contact me.

<!--[if !supportEmptyParas]--> <!--[endif]-->

Regards,

<!--[if !supportEmptyParas]--> <!--[endif]-->

Mary

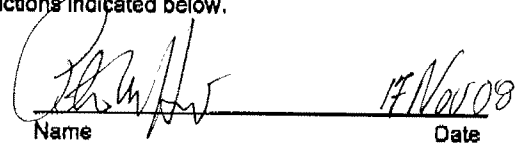
Mary N. Burtnick

Name Date

USAMRIID Approval (note, this does not constitute OPSEC or PAO approval):

Science Director:

The enclosed submission is approved for release from USAMRIID with the distribution restrictions indicated below.



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Sponsor Approval :

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( ) F: Further dissemination only as directed (classified)
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-----Original Message-----
From: Angerman, Catherine M Ms CIV USA MEDCOM USAMRIID
Sent: Tuesday, December 09, 2008 2:50 PM
To: DeShazer, David Dr CIV USA MEDCOM USAMRIID
Cc: Kenyon, Katheryn F Ms CIV USA MEDCOM USAMRIID
Subject: FW: USAMRIID Clearance Request TR-08-113

Please read the emails below. Upon completion of ANY requested changes and/or addition of disclaimers, the manuscript is cleared.

-----Original Message-----
From: USAMRMC CLEARANCES
Sent: Monday, December 08, 2008 10:22 AM
To: Angerman, Catherine M Ms CIV USA MEDCOM USAMRIID
Subject: FW: USAMRIID Clearance Request TR-08-113

All reviews are complete.

-----Original Message-----
From: Angerman, Catherine M Ms CIV USA MEDCOM USAMRIID
Sent: Monday, November 24, 2008 11:36 AM
To: USAMRMC CLEARANCES
Subject: USAMRIID Clearance Request TR-08-113

This document is cleared for all audiences for OPSEC purposes. Cleared 8 December 2008.

Judy Hoffman
Force Protection
AT/Phy.Sec./OPSEC

PAO Reviewed

Request clearance of the attached manuscript to be submitted for publication to Infection and Immunity.

Thank you,
Kate

-----Original Message-----
From: Deshazer, David Dr CIV USA USAMEDCOM
[mailto:david.deshazer@us.army.mil]
Sent: Tuesday, November 18, 2008 6:36 PM
To: Angerman, Catherine M Ms CIV USA MEDCOM USAMRIID
Subject: Re: USAMRIID Clearance Request TR-08-113

----- Original Message -----
From: "Angerman, Catherine M Ms CIV USA MEDCOM USAMRIID" <kate.angerman@us.army.mil>
Date: Tuesday, November 18, 2008 18:14
Subject: USAMRIID Clearance Request TR-08-113
To: "DeShazer, David Dr CIV USA MEDCOM USAMRIID" <David.DeShazer@amedd.army.mil>

Dr. DeShazer,

Please send an electronic copy of your manuscript, entitled "*Burkholderia mallei* tssM Encodes a Secreted Deubiquitinase That Is Expressed Inside Infected RAW 264.7 Murine Macrophages," to this Office for forwarding to MEDCOM for final clearance.

Your manuscript has been approved for clearance by the Science Director. Please reference tracking number TR-08-113 in all future correspondence.
Thank you,
Kate

INFECTION AND IMMUNITY, Apr. 2009, p. 1636–1648 Vol. 77, No. 4
0019-9567/09/$08.00+0 doi:10.1128/IAI.01339-08

Burkholderia mallei tssM Encodes a Putative Deubiquitinase That Is Secreted and Expressed inside Infected RAW 264.7 Murine Macrophages▽†

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Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322[1]; Laboratory of Zoonotic Pathogens, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840[2]; Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702-5011[3]; and Department of Microbiology, University of Georgia, Athens, Georgia 30602[4]

Received 1 November 2008/Returned for modification 8 December 2008/Accepted 12 January 2009

***Burkholderia mallei*, a category B biothreat agent, is a facultative intracellular pathogen that causes the zoonotic disease glanders. The *B. mallei* VirAG two-component regulatory system activates the transcription of ~60 genes, including a large virulence gene cluster encoding a type VI secretion system (T6SS). The *B. mallei tssM* gene encodes a putative ubiquitin-specific protease that is physically linked to, and transcriptionally coregulated with, the T6SS gene cluster. Mass spectrometry and immunoblot analysis demonstrated that TssM was secreted in a *virAG*-dependent manner in vitro. Surprisingly, the T6SS was found to be dispensable for the secretion of TssM. The C-terminal half of TssM, which contains Cys and His box motifs conserved in eukaryotic deubiquitinases, was purified and biochemically characterized. Recombinant TssM hydrolyzed multiple ubiquitinated substrates and the cysteine at position 102 was critical for enzymatic activity. The *tssM* gene was expressed within 1 h after uptake of *B. mallei* into RAW 264.7 murine macrophages, suggesting that the TssM deubiquitinase is produced in this intracellular niche. Although the physiological substrate(s) is currently unknown, the TssM deubiquitinase may provide *B. mallei* a selective advantage in the intracellular environment during infection.**

Glanders, a disease caused by *Burkholderia mallei*, is one of the oldest infectious diseases known (35, 59, 61). *B. mallei* is an obligate animal pathogen, and it cannot persist in the environment outside of its equine hosts. Infected equids can die within months, but survivors often act as carriers. Humans are accidental hosts of *B. mallei*, and the majority of cases are the result of occupational contact with infected horses. Glanders is endemic in Africa, Asia, the Middle East, and Central and South America, but it has been eradicated from North America and most of Europe through the development of an effective skin test and the slaughter of infected animals (14, 19, 26). The disease is now excluded from most countries by serological testing and quarantine. *B. mallei* is highly infectious in a laboratory setting, especially via the aerosol route (23, 25, 28, 50, 52). Diagnosis and treatment can be challenging, and no licensed vaccines are currently available. Because it is widely believed that *B. mallei* has the potential for use as a biological weapon, it has been designated as a category B select agent by the Centers for Disease Control and Prevention (44, 58).

B. mallei is a facultative intracellular pathogen that can invade, survive, and replicate in epithelial and phagocytic cell lines (8, 20, 42). This pathogen escapes the phagocytic vacuole into the cytoplasm, where it can utilize actin-based motility for intra- and intercellular spread (42, 51). Several virulence determinants of *B. mallei* have been identified and characterized by using animal models of infection, including a capsular polysaccharide, the animal pathogen-like type III secretion system ($T3SS_{AP}$), and a type VI secretion system (T6SS) (16, 46, 55). In addition, a complex quorum-sensing network and a two-component transcriptional regulatory system (VirAG) are required for maximal virulence in hamsters (46, 56). The environmental cues recognized by the VirAG two-component system are currently unknown, and overexpression of this regulatory system is currently the only way to activate transcription of the VirAG regulon in vitro (46). VirAG activates the transcription of ~60 genes, including the T6SS gene cluster (46). The *tssM* gene is located at the 3′ end of the T6SS locus, and it is activated approximately fivefold by *virAG*. TssM contains Cys and His motifs that are present in proteins of the cysteine peptidase C19 family (4). Members of this protein family include eukaryotic ubiquitin-specific proteases (USPs) that function as deubiquitinating enzymes (DUBs) (2, 32, 40, 62).

Ubiquitination is a reversible posttranslational modification that can alter a protein's stability or function (10, 41). Ubiquitin (Ub) is a 76-amino-acid protein that is highly conserved in eukaryotic organisms but is absent in bacteria. The 8.5-kDa Ub can be covalently attached to lysine residues on proteins through the action of three distinct enzymes, E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme), and E3 (Ub-protein ligase). Proteins can be monoubiquitinated or conjugated with polyubiquitin (polyUb) chains at one or more lysine residues. Ub contains seven lysine residues—K6, K11, K27, K29, K33, K48, and K63—that can be used to form polyUb chains. The

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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 21 January 2009.

type and number of Ub or polyUb chains that are attached to proteins generate distinct intracellular signals (41, 53, 60). For example, proteins that contain K48-linked polyUb chains are often targeted for destruction by the 26S proteasome, while proteins harboring K63-linked polyUb chains are often involved in intracellular signaling pathways. There are also a growing number of Ub-like proteins (Ubls) that can be post-translationally conjugated to proteins to modulate function, including SUMO, ISG15, Nedd8, and Atg8 (27, 60). DUBs and Ubl-specific proteases are enzymes that mediate the removal of Ub and Ubls from proteins and essentially reverse the fate of these tagged substrates within the cell (2, 32, 40, 62).

Recent studies have shown that several intracellular pathogenic bacteria exploit eukaryotic Ub signaling and proteolysis pathways by exporting DUBs (3, 38, 45). Bacterial DUBs appear to facilitate evasion of the host immune response and promote survival and replication in vivo. Most of the bacterial DUBs described thus far are secreted by type III secretion systems (T3SSs) and are members of cysteine protease clan CE, a clan that is distinct from the "classic" eukaryotic DUBs in cysteine protease clan CA (4, 45).

In the present study, we characterized TssM, a clan CA cysteine protease exported by *B. mallei*. We found that TssM secretion was dependent on the VirAG two-component system but did not require the cluster 1 T6SS (46) or the $T3SS_{AP}$ (55). Purified recombinant TssM (rTssM) cleaved multiple ubiquitinated substrates, and the cysteine at position 102 in rTssM was critical for DUB activity. The *tssM* gene was expressed within 1 h after *B. mallei* infection of RAW 264.7 murine macrophages, but the actual substrate of TssM is currently unknown. The potential role of this DUB in manipulating cellular functions during the host-pathogen interaction is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are described in Table 1. *Escherichia coli* strains were grown at 37°C on LB agar (Lennox L agar) or in LB broth (Lennox L broth). *B. mallei* strains were grown at 37°C on LB agar or in LB broth containing 4% glycerol (LBG). When appropriate, antibiotics were added at the following concentrations: 100 μg of ampicillin (Ap)/ml, 25 μg of streptomycin (Sm)/ml, 25 μg of chloramphenicol (Cm)/ml, 50 μg of zeocin (Zeo)/ml, 12.5 μg of tetracycline (Tc)/ml, 25 μg of kanamycin (Km)/ml, and 50 μg of trimethoprim (Tp)/ml for *E. coli* and 15 μg of polymyxin B (Pm)/ml, 5 μg of Zeo/ml, and 5 μg of Km/ml for *B. mallei*. For induction studies, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM. All manipulations with *B. mallei* were carried out in class II microbiological safety cabinets located in designated biosafety level 3 laboratories.

Gene replacement experiments with *B. mallei* SR1 were performed using the *sacB*-based vector pEx18Km. After conjugation (15, 54), the transconjugants growing on LBG plates containing Km and Pm were inoculated onto 1× M9 minimal salts (Sigma-Aldrich, St. Louis, MO) agar containing 0.4% glucose and 5% sucrose. The sucrose-resistant colonies that arose after incubation at 37°C for 4 days were screened for deletion mutations using the colony PCR procedure described below.

For the preparation of culture supernatant proteins for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, *B. mallei* (pBHR2-*virAG*) was grown at 37°C in liquid medium comprised of 3% glycerol, 1.5% Casamino Acids, 1.5% yeast extract, and Km. Before use in the medium, concentrated solutions of yeast extract and Casamino Acids were placed in dialysis membrane bags and dialyzed for 2 days against an equal volume of water, and the solution outside the dialysis bag (with compounds < 10 kDa) was removed and evaporated to dryness before being added as powder to the medium. Cultures were grown from a starting optical density at 600 nm of 0.1 for 36 h at 37°C, and cells were removed by repeated centrifugation (4,000 × *g*, 30 min). Culture supernatants were filtered through 0.22-μm-pore-size PES filters and concentrated 100-fold by ultrafiltration under pressure using an Amicon UM10 filter.

LC-MS/MS. Concentrated *B. mallei* (pBHR2-*virAG*) supernatant proteins (40 μg) in 50 mM ammonium carbonate (pH 8.2) were reduced with 10 mM dithiothreitol (DTT) at 52°C and alkylated with 50 mM iodoacetamide at 25°C in the dark for 1 h, and modified trypsin (4 μg) was added. After 16 h at 37°C, the sample was adjusted to 0.1% trifluoroacetic acid and desalted by using reversed-phase Microspin C18 columns; the peptides were then eluted in 0.1% trifluoroacetic acid–70% acetonitrile, and vacuum dried. Peptides were resuspended in 1% acetic acid–4% acetonitrile under nitrogen, loaded onto a 10-cm-by-0.075-mm column of 5-μm C18 beads, and separated with a 120-min linear gradient of acetonitrile (250 nl/min). Fractions were loaded onto a Finnigan LTQ, linear ion trap mass spectrophotometer run in automatic mode. One MS scan (100 ms) was collected, followed by eight MS/MS scans (100 ms) of the eight major peptides with a dynamic exclusion of 2 and mass gate of 2.2 Da.

DNA manipulations and introduction of plasmid DNA into *B. mallei*. Restriction enzymes, shrimp alkaline phosphatase, and T4 DNA ligase were purchased from Roche Molecular Biochemicals and used according to the manufacturer's instructions. When necessary, the End-It DNA End-Repair kit (Epicentre) was used to convert 5′- or 3′-protruding ends to blunt-ended DNA. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III kit (Qbiogene). Bacterial genomic DNA was prepared as previously described (64). Plasmids were purified from overnight cultures by using Wizard Plus SV Minipreps (Promega). The gene replacement vector pEx18Km was constructed by amplifying the Km-resistant (Km^r) gene from pCR2.1-TOPO using nptF and nptR (Table 2), digesting the resulting PCR product with SmaI, ligating the product into the unique EcoRV site in pEx18Tc, and selecting for resistance to Km and sensitivity to Tc (21). Recombinant derivatives of pEx18Km, pBHR2, and pZSV were electroporated into *E. coli* S17-1 and mated with *B. mallei* for 8 h, as described previously (15, 54). Km or Zeo were used to select for transconjugants, and Pm was used as a counterselection for *E. coli* S17-1.

PCR. The oligodeoxyribonucleotide primers used in PCRs are shown in Table 2. PCR products were fractionated by size and purified using agarose gel electrophoresis; cloned into pET200/D-TOPO, pCR2.1-TOPO, or pCR4Blunt-TOPO; and transformed into competent *E. coli* TOP10. PCR amplifications were performed in a final reaction volume of 100 μl containing 1× *Taq* PCR Master Mix (Qiagen), 1 μM oligodeoxyribonucleotide primers, and 200 ng of genomic DNA. PCR cycling was performed by using a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research, Inc.) and heated to 97°C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97°C for 30 s, 55°C for 30 s, and 72°C for 1 min) and one cycle at 72°C for 10 min. For PCR products greater than 1 kb, an additional 1 min per kb was added to the extension time. To generate blunt-ended PCR products, the 1× *Taq* PCR Master Mix was replaced with $Vent_R$ DNA polymerase (New England Biolabs) and 1× FailSafe PreMix D (Epicentre). *B. mallei* deletion mutants were identified by PCR as described above.

Site-directed mutagenesis. A pET200-*tssM* derivative in which the Cys codon (TGC) at position 102 in rTssM was changed to a Gly codon (GGC) was generated using the primers C102G-F and C102G-R (Table 2) and a QuikChange II site-directed mutagenesis kit (Stratagene). The pET200-*tssM*C102G DNA insert was sequenced on both strands to confirm the mutation.

Expression and purification of His-tagged rTssM and rTssMC102G. Plasmids pET200-*tssM* and pET200-*tssM*C102G were transformed into *E. coli* Rosetta (DE3), and the recombinant proteins were expressed and purified as previously described for the *B. mallei* recombinant HcpV5 protein (46).

Ub-AMC enzymatic assays. The 7-amidomethylcourmarin (AMC) substrates of Ub, SUMO1, SUMO2, ISG15, and Nedd8 were generated as previously described (63). The Ub-AMC assays were performed at room temperature using 1 mM Ub-AMC (A.G. Scientific, Inc.) and 100 nM rabbit Isopeptidase T (A. G. Scientific, Inc.), rTssM, and rTssMC102G. The reactions were carried out in reaction buffer containing 0.5 M HEPES, 10 mM DTT, and 1 M NaCl at pH 7.5. Reactions were monitored by following the increase in fluorescence at 460 nm (emission) and 380 nm (excitation) at 15-s intervals for 15 min using a SpectraMax M5 spectrophotometer (Molecular Devices).

In order to determine the fraction of active enzyme, Ub-VME was added as 2 and 10 molar equivalents to rTssM and rTssMC102G, followed by incubation for 30 min at 37°C. The reactions were quenched with 3× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, applied to a 10 to 20% gradient gel, and developed using Pierce Imperial Stain (Thermo Fisher Scientific, Rockford, IL). The band densities were determined for the 2 and 10 molar equivalent lanes for rTssM with 77.7 and 78.0% in the upper (binding) band, respectively.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
TOP10	General cloning and blue/white screening	Invitrogen
Rosetta (DE3)	BL21 derivative containing tRNAs for AGG, AGA, AUA, CUA, CCC, and GGA codons; Cm^r	Novagen
S17-1	Mobilizing strain with transfer genes of RP4 integrated on chromosome; Sm^r Tp^r Pm^s	49
B. mallei		
ATCC 23344	Type strain; isolated in 1944 from a human case of glanders; Pm^r Km^s Zeo^s	39
SR1	ATCC 23344 sucrose-resistant derivative; Δ(BMAA0437-BMAA0497)	54
DDA0729-1	SR1 derivative; Δ*tssM*::GFP	This study
DDA0739	SR1 derivative; Δ*tssE*	46
DDA0740	SR1 derivative; Δ*tssD*	46
DDA0742	SR1 derivative; Δ*hcp1*	46
DDA0743	SR1 derivative; Δ*tssB*	46
DDA0737	SR1 derivative containing a 744-bp StuI in-frame deletion internal to *vgrG1*; Δ*vgrG1*	This study
DDA1533	ATCC 23344::pZSV-*bsaZ*; Zeo^r	This study
Plasmids		
pCR2.1-TOPO	3,931-bp TA vector; pMB1 *oriR*; Km^r Ap^r	Invitrogen
pCR2.1-29THS	pCR2.1-TOPO containing 1,424-bp PCR product generated with 29THS3 and 29THS2	This study
pCR2.1-A0737	pCR2.1-TOPO containing 1,662-bp PCR product generated with 37-up and 37-dn	This study
pCR2.1-ΔA0737	pCR2.1-A0737 derivative containing a 744-bp StuI in-frame deletion internal to *vgrG1*	This study
pCR2.1-rUb	pCR2.1-TOPO containing a 243-bp PCR product generated with rUb-up and rUb-dn using pUb-S27a as a template	This study
pET200/D-TOPO	5,741-bp directional cloning and expression vector; pBR322 ori; Km^r	Invitrogen
pET200-*tssM*	pET200/D-TOPO containing 858-bp PCR product generated with tssM-up3 and tssM-dn2	This study
pET200-*tssM*C102G	pET200-*tssM* derivative in which the Cys codon (TGC) at position 102 in rTssM has been changed to a Gly codon (GGC)	This study
pCMV-SPORT6	4,396-bp mammalian expression vector; pBR322 ori; Ap^r	Invitrogen
pUb-S27a	pCMV-SPORT6 containing a 572-bp SalI-NotI *Homo sapiens* Uba80 (RPS27a) cDNA insert; ATCC 9890377; MGC 61435; IMAGE 5531043	ATCC
pCMV-3Tag-6	5,241-bp mammalian expression vector with N-terminal 3x-FLAG tag; pUC ori; Ap^r	Stratagene
pFLAG-Ub	pCMV-3Tag-6 containing BamHI-HindIII insert from pCR2.1-rUb	This study
pRD02	pTSV3 derivative containing an internal 313-bp PCR product from the ATCC 23344 *bsaZ* gene; Tp^r	55
pBHR2	Broad-host-range vector; Km^r	46
pBHR2-*virAG*	pBHR2 derivative containing *B. mallei virAG* genes	46
pBHR2-*tssM*	pBHR2 containing 1,424-bp BamHI fragment from pCR2.1-29THS	This study
pCR4Blunt-TOPO	3,956-bp positive selection cloning vector; pUC ori; Km^r Ap^r	Invitrogen
pCR4-Δ*tssM*	pCR4Blunt-TOPO containing a 632-bp fragment generated by joining the SEW1/SEW2 and SEW3/SEW4 PCR products via SOE-PCR	This study
pQBI T7-GFP	GFP expression vector; Ap^r	Quantum Biotech
pCR4-Δ*tssM*::GFP	pCR4-Δ*tssM* containing 762-bp BamHI-XbaI fragment from pQBI T7-GFP	This study
pEx18Tc	Gene replacement vector; *lacZ*α; oriT; *sacB*; Tc^r	21
pEx18Km	pEx18Tc derivative containing the 1,003-bp Km^r PCR product generated with nptF and nptR from pCR2.1-TOPO; Km^r Tc^s	This study
pΔ*tssM*::GFP	pEx18Km derivative containing the 1,380-bp EcoRI fragment from pCR4-Δ*tssM*::GFP	This study
pΔA0737	pEx18Km derivative containing XbaI-SpeI fragment from pCR2.1-ΔA0737 in XbaI site	This study
pZSV	Mobilizable suicide vector; Zeo^r	9
pZSV-*bsaZ*	pZSV derivative containing EcoRI fragment from pRD02	This study

[a] Superscripts: r, resistant; s, susceptible.

Enzyme kinetics were determined in assay buffer (50 mM Tris-HCl [pH 7.5], 2 mM DTT, and 10 μg of ovalbumin/ml) with increasing amounts of Ub-AMC at 37°C and monitored at 440 nm (emission) and 340 nm (excitation) for 100 s after the addition of rTssM. Competitive inhibitor experiments were performed using 498 nM Ub-AMC and increasing concentrations of free Ub or Nedd8 at 37°C. Reactions were initiated with the addition of 426 pM rTssM and monitored by using an Aminco-Bowman Series 2 luminescence spectrometer.

For enzyme assays using K48- and K63-linked Ub and Di-SUMO substrates, K48- and K63-linked Ub dimers and polyubiquitin chains, Di-SUMO-2, and Di-SUMO-3 (750 ng; Boston Biochem, Cambridge, MA) were incubated with 100 nM IsoT, rTssM, and rTssMC102G at 37°C for 30 min in a reaction buffer containing 0.5 M HEPES, 10 mM DTT, and 1 M NaCl at pH 7.5. His_6-$SENP1_{CD}$, a SUMO-specific protease 1 catalytic domain (250 nM; Boston Biochem), was used a positive control for the SUMO substrates. The reactions were mixed with an equal volume of 2× Tris-glycine-SDS sample buffer and boiled for 5 min. The samples were loaded onto a 10 to 20% Tris glycine gel, and the proteins were separated by electrophoresis using 1× Tris-glycine-SDS buffer and stained with a colloidal blue staining kit.

Cleavage and immunoblot analysis of eukaryotic proteins tagged with 3x-FLAG-Ub. The human Ub gene (Uba80) was PCR amplified from pUb-S27a using the oligodeoxyribonucleotide primers rUb-up and rUb-dn (Table 2). The resulting PCR product was directionally cloned into the mammalian expression vector pCMV-3Tag-6, generating pFLAG-Ub (Table 1). The 293T human embryonic kidney cell line was cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum at 37°C with 5% CO_2. The 293T cells were seeded onto a six-well

TABLE 2. Oligodeoxyribonucleotide primers used in this study

Primer	Primer sequence (5′ to 3′)	Target
tssM-up3	CACCATGCGCCGCCCGACGATCGAA	*tssM*
tssM-dn2	TCATCGATAGATCGCGATTTC	*tssM*
C102G-F	CTCGACGGCGCCTCGGGCTTCATCAACA CGGCG	*tssM*
C102G-R	CGCCGTGTTGATGAAGCCCGAGGCGCCG TCGAG	*tssM*
29THS3	GGATCCATGAACGCTCGACGTCCGGC	*tssM*
29THS2	GGATCCTCATCGATAGATCGCGATTTC	*tssM*
SEW1	GAATTCGCGCCCCCGAC	*tssM*
SEW2	GGATCCTTGGATGCTCTAGATTCGAGTT CGATCGTC	*tssM*
SEW3	TCTAGAGCATCCAAGGATCCCGTTCAAC CCCGCTAAC	*tssM*
SEW4	GAATTCGACGGCGCGAC	*tssM*
37-up	CATTCGACGTCGTCGACATC	*vgrG1*
37-dn	GCAGCGTCGTCAGGAAGTTC	*vgrG1*
rUb-up	GGATCCATGCAGATTTTCGTGAAAAC	Uba80
rUb-dn	AAGCTTTTAACCACCACGAAGTCTC	Uba80
nptF	CCCGGGTTTTATGGACAGCAAGCG	npt2
nptR	CCCGGGTCAGACACGGAAATGTTGAATA	npt2

plate at a concentration of 4×10^5 cells/well, grown for 24 h, and transfected with pCMV-3Tag-6 and pFLAG-Ub using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 48 h posttransfection, the spent medium was removed, and the cells were resuspended in fresh DMEM. The cells were harvested by centrifugation (2,200 × *g*, 5 min) at 4°C. The supernatants were removed, and the cells were washed with phosphate-buffered saline (PBS). After centrifugation, the cells were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% Triton X) containing phosphatase inhibitor cocktail (P5726; Sigma, St. Louis, MO) and incubated on ice for 30 min. The cellular lysates were centrifuged for 30 min, and the protein concentrations of the supernatants were determined by using a BCA protein assay kit (Pierce, Rockford, IL). Proteins (30 μg) obtained from the pFLAG-Ub transfected cells were incubated with 1 μM rTssM and 1 μM rTssMC102G at 37°C for 1 h in a reaction buffer containing 0.5 M HEPES, 10 mM DTT, and 1 M NaCl at pH 7.5. The reactions were mixed with an equal volume of 2× Tris-glycine-SDS sample buffer and boiled for 2 min. The samples were loaded onto a 10 to 20% Tris-glycine gel, and the proteins were separated by electrophoresis using 1× Tris-glycine-SDS buffer and transferred to Invitrolon polyvinylidene difluoride (Invitrogen) using a XCell SureLock apparatus (Invitrogen). The membranes were blocked with 3.5% skim milk powder (EMD Chemicals, Inc.)–0.1% Tween 20 (Sigma), incubated with a 1:5,000 dilution of murine anti-FLAG M2 monoclonal antibody (Sigma) and a 1:5,000 dilution of horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin G (IgG; H+L) (KPL), and developed with TMB membrane peroxidase substrate (KPL). The membranes were also reacted with a 1:2,500 dilution of nickel-labeled HRP (Ni-HRP) and developed as described above to detect rTssM and rTssMC102G.

Production of polyclonal antisera against rTssM. Ten BALB/c mice were injected intraperitoneally with 50 μg of rTssM in complete Freund adjuvant (Sigma). Three weeks after injection, the mice were boosted intraperitoneally with 50 μg of rTssM in incomplete Freund adjuvant (Sigma). A second boost containing 50 μg of rTssM in incomplete Freund adjuvant was given intraperitoneally 3 weeks after the first boost for a total of three injections. At 6 weeks after the final boost, anesthetized mice were bled retroorbitally, and the leukocytes were removed by centrifugation. The serum samples were pooled and used for immunoblotting experiments.

Immunoblotting with murine anti-rTssM sera. Wild-type and mutant *B. mallei* strains were grown for 8 h in 14-ml snap-cap tubes containing 3 ml of LBG for rTssM immunoblotting experiments. One-milliliter volumes were transferred to microcentrifuge tubes, and cell pellets were obtained after centrifugation at 14,000 rpm for 2 min at 25°C. Cell pellets were washed once with 1× PBS, resuspended in 1 ml of 1× Tris-glycine-SDS sample buffer (Invitrogen), boiled for 10 min, and stored at 4°C. Three microliters of sample was loaded onto a 10 to 20% Tris gel, and proteins were separated by electrophoresis using 1× Tris-glycine-SDS running buffer. Supernatants were filter sterilized through 0.45-μm-pore-size Millex HV filters (Millipore), and 200 μl was precipitated at −20°C for 30 min using 10% trichloroacetic acid. Protein pellets were collected by centrifugation (14,000 rpm, 10 min) and washed once with cold acetone. The protein pellets were resuspended in 1× Tris-glycine-SDS sample buffer and processed as described above. Proteins were transferred to Invitrolon polyvinylidene difluoride by using a XCell SureLock apparatus (Invitrogen). The membranes were blocked with 3.5% skim milk powder (EMD Chemicals, Inc.)–0.1% Tween 20 (Sigma), incubated with a 1:5,000 dilution of anti-rTssM murine polyclonal sera antibody, and washed three times with blocking buffer. The membranes were then reacted with a 1:5,000 dilution of peroxidase-labeled goat anti-mouse IgG (H+L; KPL), washed three times with blocking buffer, washed once with PBS, and then developed with TMB membrane peroxidase substrate (KPL).

Immunofluorescence staining and confocal microscopy. The murine macrophage cell line RAW 264.7 (ATCC TIB-71) was obtained from the American Type Culture Collection (ATCC). RAW 264.7 cells ($\sim 2 \times 10^5$) were grown overnight on 12-mm glass coverslips (Fisher Scientific) at 37°C under an atmosphere of 5% CO_2. Macrophages were infected with *B. mallei* DDA0729-1 (Δ*tssM*::GFP) at an multiplicity of infection (MOI) of 40, utilizing a modified Km protection assay as previously described (8, 9). Briefly, at various times postinfection, monolayers were fixed in 2.5% paraformaldehyde for 15 min, followed by extensive washing in PBS. Cells were then incubated with Alexa Fluor 647 phalloidin (1:100; Invitrogen) in SS-PBS for 45 min. After extensive washing in PBS, coverslips were mounted onto glass slides using Mowiol. Laser confocal microscopy was performed with a Zeiss 510 META confocal imaging system equipped with an Ar, HeNe laser on an inverted Axiovert 200 M microscope using a ×63 oil objective lens (Carl Zeiss MicroImaging, Inc.). Images of 1,024 × 1,024 pixels were acquired by using Zeiss 510 Meta software (Carl Zeiss).

Macrophage survival assays. Cells were maintained in DMEM (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Invitrogen) and a standard mixture of antibiotics (100 U of penicillin/ml, 100 μg of streptomycin/ml, and 250 μg of amphotericin B/ml) at 37°C under an atmosphere of 5% CO_2. Bacterial uptake and survival were measured by using modified Km protection assays as previously described (8, 9). In brief, bacterial suspensions were added in triplicate onto RAW 264.7 cells at an MOI of 1 or 10. The monolayers were incubated with the bacteria at 37°C under an atmosphere of 5% CO_2 for 1 h and then washed twice with Hanks balanced salt solution (Invitrogen) to remove extracellular bacteria. Infected RAW 264.7 cells were then incubated in fresh DMEM-10 containing 250 μg of kanamycin/ml (with or without 200 μg of aminoguanidine/ml) to suppress the growth of residual extracellular bacteria. Monolayers were lysed at 3 and 24 h postinfection with 0.25% (vol/vol) Triton X-100, and serial dilutions of the lysates were plated onto brucella agar (Difco) supplemented with 4% glycerol and incubated at 37°C for 48 h. Bacterial loads on plates were counted.

Cytokine assays. Culture supernatants were harvested from infected RAW 264.7 cell monolayers at various time points and sterilized using 0.45-μm-pore-size Millex syringe-driven filter units (Millipore Corp.). Aliquots of each were plated onto BB4G agar and incubated at 37°C for at least 72 h to confirm sterility. The sterilized supernatants were then assayed for the production of tumor necrosis factor alpha, interleukin-1 (IL-1), IL-6, and RANTES using murine SearchLight custom multiplex arrays (Pierce).

Animal studies. Three groups of female Syrian hamsters (five per group) were infected intraperitoneally with 10, 100, and 1,000 bacteria for each strain of *B. mallei* examined. Mortality was recorded daily for 5 days. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

RESULTS

TssM is secreted when the *virAG* two-component regulatory system is overexpressed. We recently utilized SDS-PAGE and LC-MS/MS to identify two *B. mallei* T6SS proteins, Hcp1 and VgrG1, present in culture supernatants (46). A protein of ~50 kDa was also found in culture supernatants when *virAG* was overexpressed, but it was not positively identified. The goal of this investigation was to identify and characterize this protein. We performed more sensitive shotgun LC-MS/MS analyses and found that, like Hcp1, TssM (BMAA0729) was present at much higher levels in culture supernatants when *virAG* was

overexpressed (data not shown). In fact TssM and Hcp1 were the two most abundant proteins in these culture supernatants, representing more than half the total protein. SDS-PAGE and LC-MS/MS of the culture supernatants did not show evidence of major cell lysis nor any evidence of leakage of periplasmic proteins, suggesting that TssM is secreted in vitro by *B. mallei* when *virAG* is overexpressed.

Analysis of the annotated *tssM* gene suggests an alternate start site. The *B. mallei* ATCC 23344 genome sequence was published in 2004 (39), and *tssM* (BMAA0729) was annotated as a 1,980-bp (bp) gene encoding a 659-amino-acid protein of ~70 kDa (Fig. 1A and B). This is significantly larger than the ~50-kDa protein detected in *B. mallei* supernatants when *virAG* is overexpressed (46). In addition, the TssM peptides detected by LC-MS/MS were present only in the C-terminal half of the putative 70-kDa protein (between amino acids 378 and 625). We considered the possibility that *tssM* was incorrectly annotated and used a downstream start codon, especially since its predicted start codon preceded the stop codon of upstream *icmF1* gene (BMAA0730) by ~20 bp. Several alternative start codons were identified, and the overall coding potential of the annotated *tssM* gene was assessed by using the GeneMark.hmm program (Fig. 1B) (33). An open reading frame with high coding potential was identified between nucleotides 1126 and 1980 using typical and atypical hidden Markov models and hidden Markov models based on genes predicted in the *B. mallei* genome (Fig. 1B). The predicted start codon for this gene is TTG, which is rarely used in *B. mallei* (39). Furthermore, the encoded protein of this gene is only ~30 kDa. A potential GTG start codon at position 625 also encodes a protein that is too small to be TssM (Fig. 1B). The actual *tssM* start codon is likely the ATG at nucleotide position 556, since this predicts *tssM* encodes a protein of ~50 kDa (474 amino acids). This start site was confirmed by immunoblot analysis and complementation studies (see below). Thus, the original annotation of *tssM* (39) incorrectly added 555 bp to the 5′ end of the gene and an additional 20 kDa to the predicted size of TssM. This 555-bp region had very low coding potential and could contain transcriptional regulatory signals.

TssM contains Cys and His box motifs conserved in cysteine peptidase family C19. The predicted TssM amino acid sequence was used to query the National Center for Biotechnology Information's conserved domain database (34) and was found to possess a peptidase C19E domain (cd02661) at the C terminus. Peptidase C19 family proteins contain eukaryotic cysteine peptidases that remove ubiquitin molecules from polyubiquinated peptides by cleavage of isopeptide bonds. The TssM protein contains the conserved Cys and His box motifs that are present in peptidase family C19 proteins, including the Asn, Cys, His, and Asp residues that are critical for catalysis (Fig. 1C). The N-terminal half of TssM does not possess a conserved domain, nor does it show >40% amino acid identity to any proteins in the nr database except those present in closely related *Burkholderia* spp. It does contain, however, a stretch of ~120 amino acids rich in Ala and Pro residues (Fig. 1C). The functional significance of this region is currently unknown, but the putative catalytic domain at the C terminus of TssM suggests that it may function as a DUB.

Cloning, expression, and purification of rTssM and production of polyclonal rTssM antiserum. The region of *tssM* containing the highest predicted coding potential, residues 191 to 474 (Fig. 1B), was cloned into a T7 promoter expression vector, and the His-tagged recombinant protein was purified by immobilized metal affinity chromatography. This recombinant protein, termed rTssM, contained the C-terminal 284 residues of the TssM protein, including the putative catalytic domain (Fig. 1C). The codon encoding the conserved Cys at position 102 in rTssM (residue 292 in wild-type TssM) was mutated to encode a Gly, and the resulting protein, rTssMC102G, was purified in the same manner. Finally, rTssM was used to immunize BALB/c mice and polyclonal antisera were obtained and pooled (see Materials and Methods).

rTssM, but not rTssMC102G, exhibits deubiquitinating activity in vitro. In order to determine whether rTssM functions as a DUB, we performed enzymatic assays using the fluorogenic substrate ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC) (12). rTssM and isopeptidase T (IsoT), a Ub-specific protease, were incubated with Ub-AMC, and both rapidly released fluorescent AMC (Fig. 2A). On the other hand, rTssMC102G could not hydrolyze Ub-AMC, and no fluorescence was observed above that of the negative control (Fig. 2A). Michaelis-Menten parameters were determined, and rTssM hydrolyzed Ub-AMC with a k_{cat} of 1.7 s^{-1}, a K_m of 175 nM, and a k_{cat}/K_m of 10^7 M^{-1} s^{-1} (Fig. 2B). We also examined the specificity of rTssM by testing its ability to cleave other fluorogenic substrates. No activity was observed against SUMO1-AMC, SUMO2-AMC, or ISG15-AMC, but minor activity was observed against Nedd8-AMC (data not shown). Because Nedd8 is 60% identical to Ub, we compared the ability of free Nedd8 and free Ub to competitively inhibit Ub-AMC cleavage by rTssM. Figure 3 demonstrates that Ub is an efficient competitive inhibitor of this reaction (K_i = 2.1 μM) and that Nedd8 is a relatively poor inhibitor (K_i = 59 μM). This result suggests that Ub, not Nedd8, is the preferred substrate of rTssM. Furthermore, the hexapeptide LRLRGG did not inhibit Ub-AMC cleavage by rTssM, suggesting that rTssM recognizes the entire Ub molecule rather than just the C terminus (data not shown).

To characterize further the activity of rTssM, we studied its activity against additional Ub substrates. Di-ubiquitin (Ub2) substrates were used to determine the ability of rTssM to cleave K48 and K63 linkages between ubiquitin molecules. IsoT and rTssM cleaved K48-linked Ub2 and K63-linked Ub2 to monomeric Ub, but rTssMC102G failed to cleave either substrate (Fig. 4). Similar results were obtained using K48- and K63-linked polyubiquitin chains (Ub1-7) as substrates (data not shown). In comparison, rTssM could not cleave dimers of SUMO-2 and SUMO-3 (data not shown). Taken together, the results demonstrate that rTssM is a specific and efficient DUB and the cysteine at position 102 is critical for its enzymatic activity.

rTssM cleaves FLAG-tagged Ub from host proteins. We next determined whether eukaryotic proteins posttranslationally modified by Ub were substrates for rTssM. The human Ub gene was cloned into a mammalian expression vector possessing an epitope tagging system in which three copies of the FLAG tag (DYKDDDDK) were added to the N terminus of Ub. 293T cells were transiently transfected with the 3x-

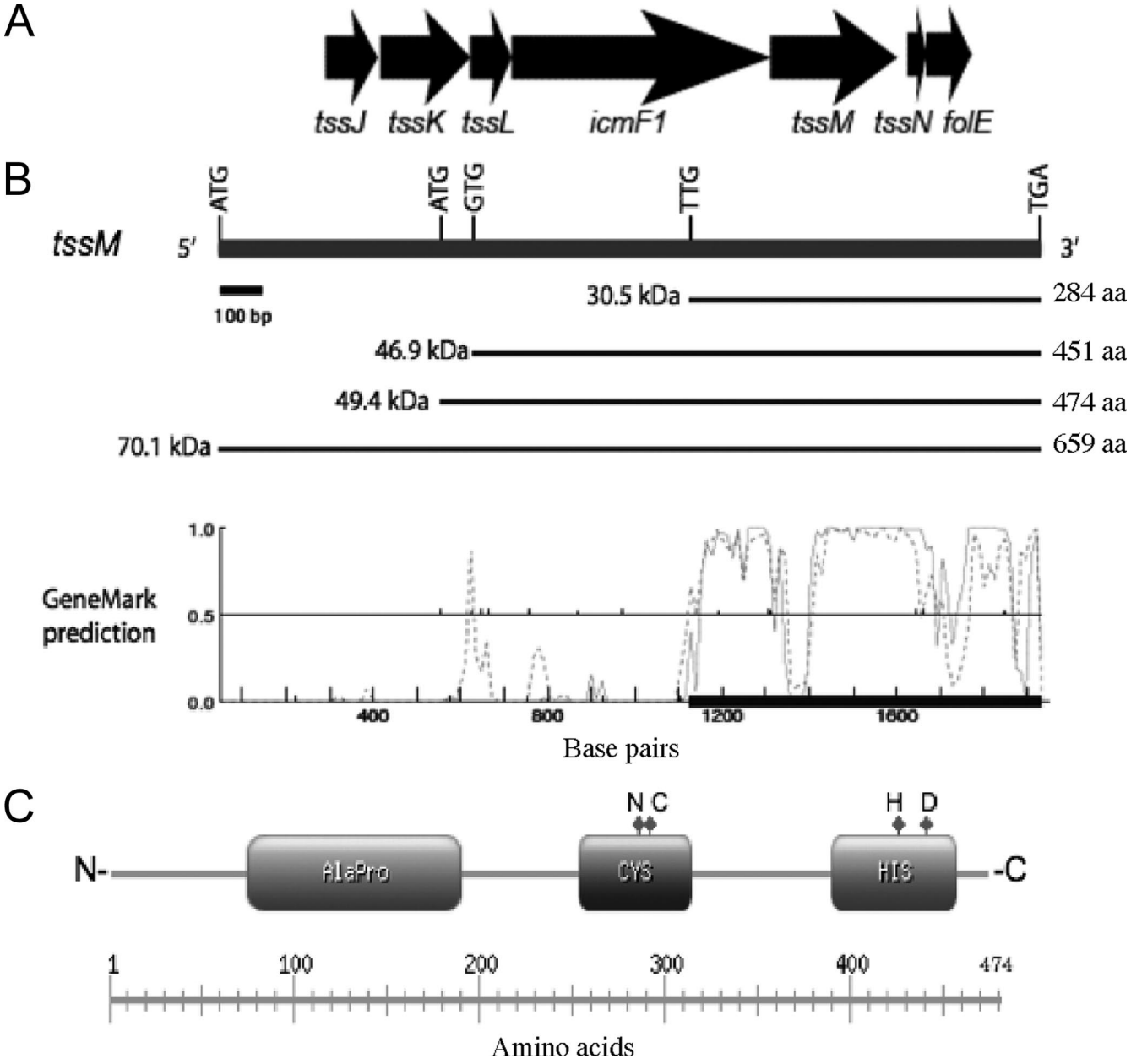

FIG. 1. Schematic representation of the *B. mallei* ATCC 23344 *tssM* gene and protein (BMAA0729). (A) *tssM* and flanking genes. *tssJ*, *tssK*, *tssL*, and *icmF1* encode T6SS proteins, whereas *tssM*, *tssN*, and *folE* encode proteins that are not associated with T6SSs in other bacteria (46, 48). (B) Alternate translation initiation sites of the *tssM* gene versus the one originally proposed (39). The 1,980-bp *tssM* gene is shown as a long black horizontal bar and putative alternate translation initiation sites (ATG, GTG, and TTG) are indicated. The sizes and locations of the proteins encoded using the alternate translation initiation sites are shown as thin black lines. Immediately below is a graphical output from GeneMark.hmm for *tssM* using a training set derived from genes on *B. mallei* ATCC 23344 chromosome 2. The solid and dashed traces indicate the coding potential calculated using the typical and atypical Markov chain models of coding DNA, respectively. The thick black horizontal bar indicates the location of an open reading frame with the highest coding potential predicted by GeneMark.hmm. The numbers on the vertical axis represent the coding potential, and the numbers on the horizontal axis represent the nucleotide position. The ticks extending above the horizontal line bisecting the graph indicate all possible alternate start codons. The scale bar represents 100 bp. (C) Schematic representation of the TssM protein and its domain structure. The protein is shown as a thin gray line with rounded rectangles showing sequence features and motifs, including the Ala- and Pro-rich N-terminal region and the C-terminal Cys and His box motifs. The Asn (N), Cys (C), His (H), and Asp (D) residues that are critical for catalysis of family C19 proteins are also shown. The scale at the bottom shows the amino acid positions in the TssM.

FLAG-Ub construct and cultured for 48 h to allow expression and conjugation of the 3x-FLAG-Ub to host proteins. The cells were lysed, and the proteins were incubated with purified rTssM and rTssMC102G to allow cleavage of the 3x-FLAG-Ub from the proteins. The reaction products were separated by SDS-PAGE, and immunoblot analysis was performed with a commercially available α-FLAG monoclonal antibody (Fig. 5). As expected, 293T cells transfected with the 3x-FLAG control

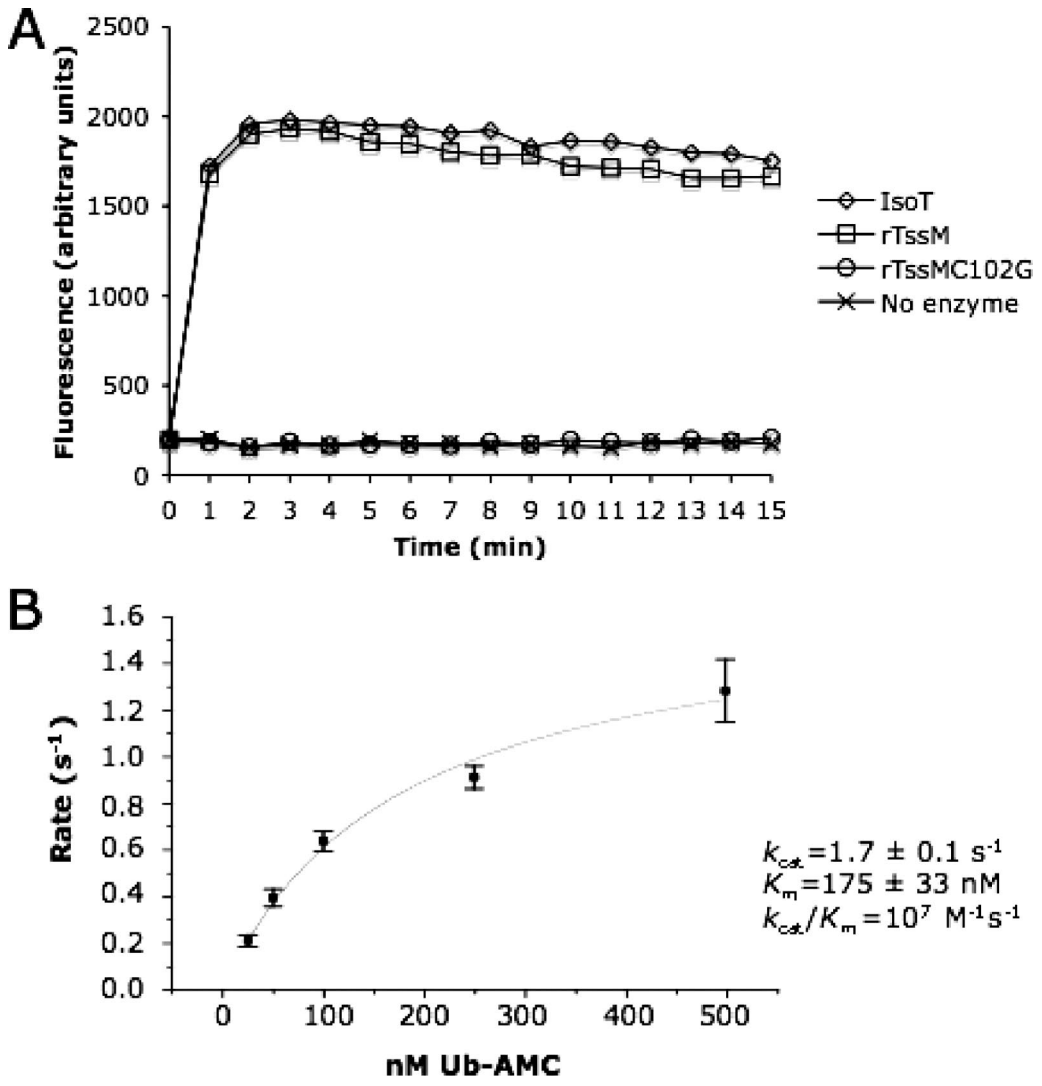

FIG. 2. Ub-AMC hydrolysis by rTssM. (A) IsoT, rTssM, and rTssMC102G were incubated with Ub-AMC, and their enzymatic activity was determined by the release of fluorescent AMC. The no enzyme control is the amount of background fluorescence emitted by Ub-AMC only. The experiment was performed on two separate occasions in triplicate and a representative result is shown. (B) Michaelis-Menten plot showing the reaction rate of rTssM with an increasing concentration of Ub-AMC. Shown are the mean values of a representative experiment with triplicate measurements ± the standard deviations. Kinetic parameters for the reaction are also shown. The activity of rTssM was 78% and was determined by titration with excess Ub-vinylmethylester.

vector did not contain any FLAG-tagged proteins. Cells transfected with the 3x-FLAG-Ub construct, however, contained numerous proteins conjugated to FLAG-tagged Ub (Fig. 5). The α-FLAG antibody reacted with unconjugated 3x-FLAG-Ub (~14 kDa) and with proteins decorated with 3x-FLAG-Ub (~30 kDa to >180 kDa). rTssM was able to remove almost all of the 3x-FLAG-Ub from these proteins (Fig. 5, wild type [WT]), while rTssMC102G could not cleave the FLAG-tagged Ub from these proteins (Fig. 5, C102G). The Ni-HRP blot at the bottom of Fig. 5 shows that similar amounts of rTssM and rTssMC102G were added to the DUB reactions. The results clearly demonstrate that rTssM can efficiently remove Ub from eukaryotic proteins with little specificity for the molecular nature of Ub attachment (monoubiquitination, multiubiquitination, or polyubiquitination). Furthermore, the cysteine at position 102 was essential for this DUB activity.

Secreted TssM is processed at several distinct sites. In order to determine the size of native TssM, we reacted murine polyclonal rTssM antisera with cell-associated and secreted proteins produced by *B. mallei* harboring *virAG* in trans. The cell-associated TssM migrated as a single protein of ~50 kDa, which is consistent with the ATG at nucleotide position 556 being the *tssM* start codon (Fig. 1B). Interestingly, the secreted TssM migrated as a ~50-kDa protein and several distinct protein bands of lower molecular mass (Fig. 6A). In order to rule out the possibility that these lower molecular mass proteins were due to unrelated cross-reactive proteins present in the supernatant, a *B. mallei* Δ*tssM* strain was constructed (see Fig. S1 in the supplemental material). The rTssM antisera did not react with any supernatant proteins from the Δ*tssM* strain, suggesting that the lower-molecular-mass proteins in the wild-type strain were processed derivatives of ~ 50-kDa TssM (Fig.

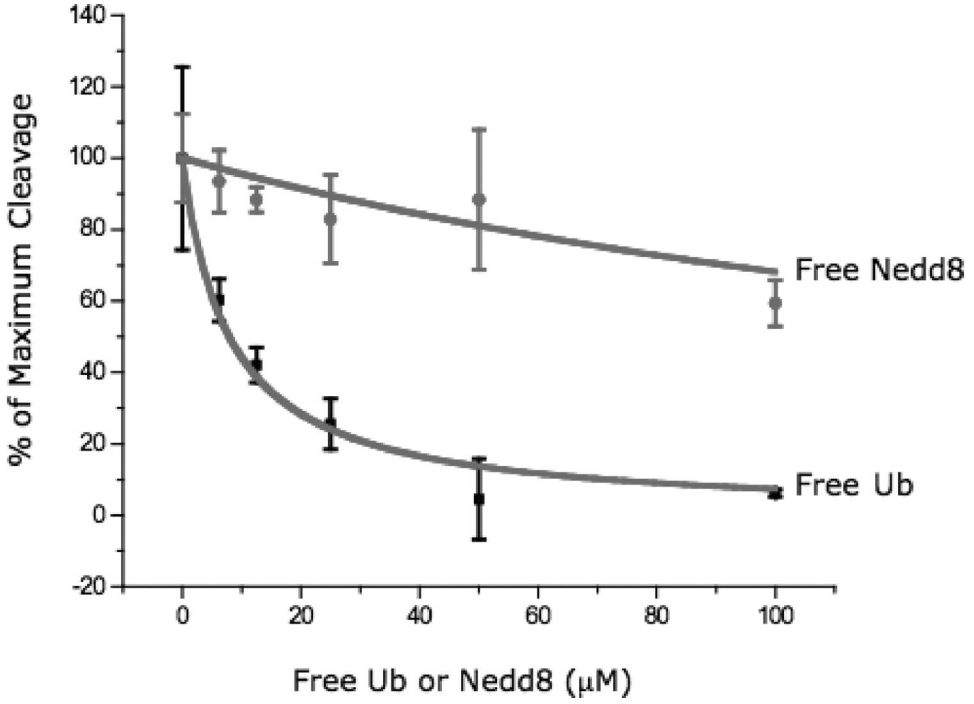

FIG. 3. Inhibitory effect of free Ub or Nedd8 on Ub-AMC cleavage by rTssM. The K_i for Ub was 2.1 μM and the K_i for Nedd8 was 59 μM. Shown are the mean values of a representative experiment with triplicate measurements ± the standard deviations.

6B). These results indicate that secreted TssM, but not cell-associated TssM, was processed at several defined sites. An in silico analysis of TssM did not identify a signal peptide cleavage site (17) or a twin-arginine translocation (Tat) cleavage

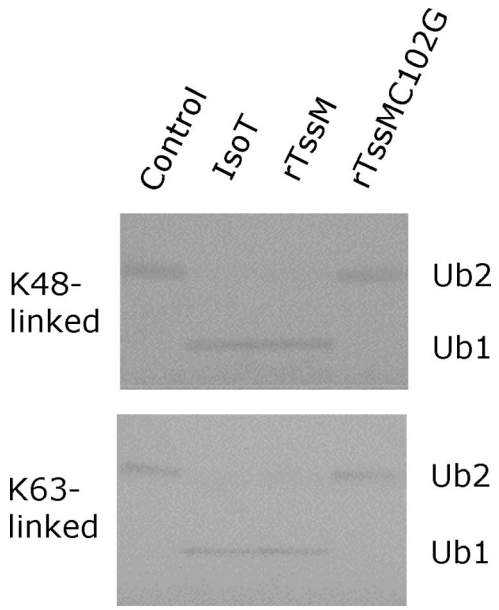

FIG. 4. rTssM, but not rTssMC102G, can cleave K48- and K63-linked Ub dimers (Ub2) into monomers (Ub1). IsoT, rTssM, and rTssMC102G were incubated with K48-linked Ub2 (top) and K63-linked Ub2 (bottom) and the reaction products were separated by SDS–10 to 20% PAGE. After electrophoresis, the gels were stained with colloidal Coomassie blue. The Ub2 substrate alone served as a negative control, and IsoT was utilized as a positive DUB control.

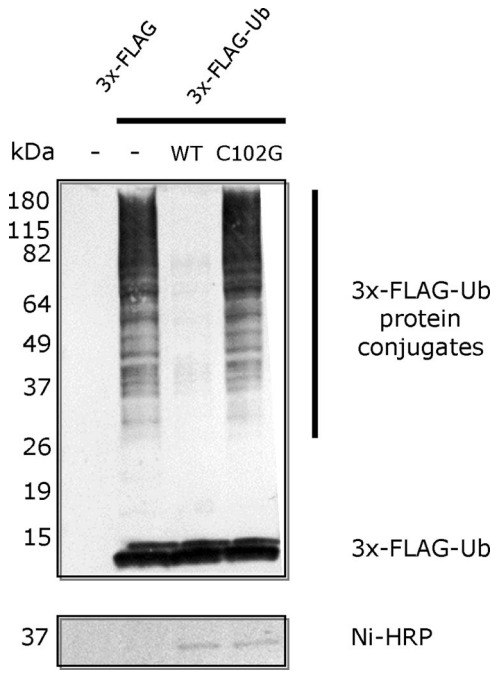

FIG. 5. Cleavage and immunoblot analysis of 293T proteins labeled with FLAG-tagged Ub. 293T cells were transfected with a 3x-FLAG vector and a 3x-FLAG-Ub construct, cultured for 48 h, and lysed. The extracted proteins (30 μg) were incubated at 37°C for 1 h with 1 μM rTssM (wild-type [WT]) and 1 μM rTssMC102G (C102G) and analyzed by immunoblot using an anti-FLAG monoclonal antibody. Cellular proteins not incubated with rTssM or rTssMC102G are designated with a negative sign (−). The migration of free 3x-FLAG-Ub and 3x-FLAG-Ub protein conjugates (vertical line) are shown to the right. The blot at the bottom was incubated with Ni-HRP and shows that His-tagged rTssM and His-tagged rTssMC102G were added in similar quantities to the DUB reactions.

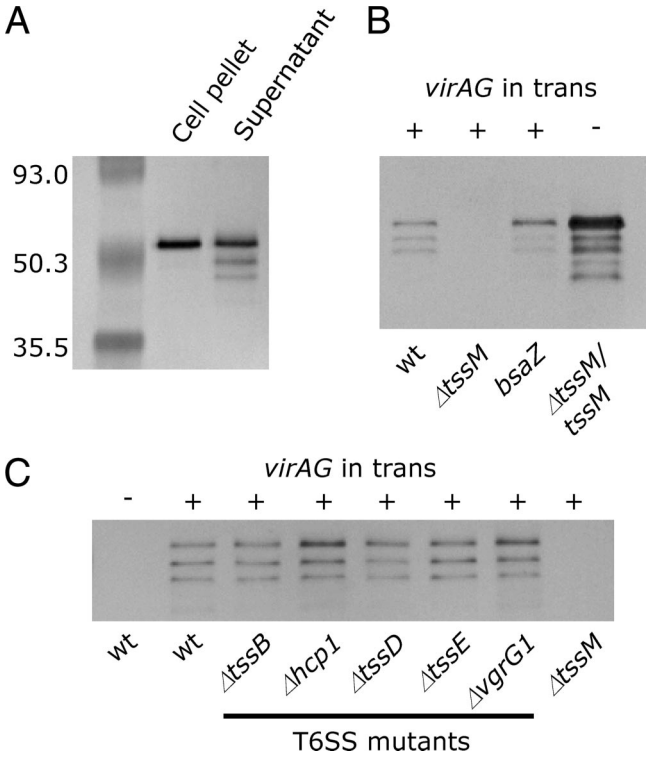

FIG. 6. Immunoblot analysis of TssM. Proteins were separated by SDS-PAGE, and immunoblots were reacted with murine polyclonal rTssM antisera and HRP-labeled goat anti-mouse IgG (H+L). (A) Cell-associated and supernatant proteins from SR1(pBHR2-*virAG*). (B) Supernatant proteins from wild type (wt), SR1(pBHR2-*virAG*); Δ*tssM*, DDA0729-1(pBHR2-*virAG*); Δ*bsaZ*, DDA1533(pBHR2-*virAG*); Δ*tssM*/*tssM*$^+$, DDA0729-1(pBHR2-*tssM*) (C), Supernatant proteins from wild type (wt) (−), SR1(pBHR2); wild type (+), SR1(pBHR2-*virAG*); Δ*tssB*, DDA0743(pBHR2-*virAG*); Δ*hcp1*, DDA0742(pBHR2-*virAG*); Δ*tssD*, DDA0740(pBHR2-*virAG*); Δ*tssE*, DDA0739(pBHR2-*virAG*); Δ*vgrG1*, DDA0737(pBHR2-*virAG*); and Δ*tssM*, DDA0729-1(pBHR2-*virAG*). The apparent molecular masses, in kilodaltons, of Bio-Rad low-range prestained SDS-PAGE standards are shown in panel A. Strains that contain pBHR2-*virAG* in *trans* are designated with a "+"; those that contain pBHR2 or pBHR2-*tssM* in *trans* are designated with a "−" in panels B and C. The five strains that harbor mutations in the T6SS gene cluster are underlined in panel C.

site (7, 43), suggesting that these signal peptidases are not involved in TssM processing. Furthermore, the full-length ~50-kDa protein was present in the culture supernatant, which indicated that proteolytic processing is probably not required for TssM secretion (Fig. 6A).

The *B. mallei* T3SS$_{AP}$ and the cluster 1 T6SS are not required for TssM secretion. *B. mallei* ATCC 23344 contains two T3SS gene clusters: a plant pathogen-like cluster (T3SS$_{PP}$) and the T3SS$_{AP}$ (55). Gene loss, inactivation, and rearrangement have occurred on a large scale during the evolution of *B. mallei* from the melioidosis pathogen *Burkholderia pseudomallei* (22, 31, 39, 57). While the T3SS$_{AP}$ gene cluster is located immediately downstream of *tssM* in *B. pseudomallei* (47), in *B. mallei* it has been rearranged to a new location (61). Given the physical linkage of *tssM* and the T3SS$_{AP}$ gene cluster in the direct ancestor of *B. mallei*, we performed an immunoblot on the supernatant proteins from a T3SS$_{AP}$ mutant (Δ*bsaZ*) to determine whether TssM was present (Fig. 6B). *B. mallei* DDA1533 contains an insertional mutation in the *bsaZ* allele (Table 1), which is a homologue of the *Salmonella enterica* serovar Typhimurium *spaS* gene. SpaS encodes a putative inner-membrane component of the *S. enterica* SPI1 T3SS (13). The insertional inactivation of the *bsaZ* allele, which is an essential component of the secretion apparatus, would likely exert a polar effect on the T3SS$_{AP}$ and disrupt effector molecule secretion. In fact, the 50% lethal dose (LD$_{50}$) of DDA1533 was ~10^5 CFU in hamsters via the intraperitoneal route of infection (data not shown). The LD$_{50}$ of ATCC 23344, on the other hand, is ~10 CFU (55). The immunoblot result clearly demonstrated that the Δ*bsaZ* strain is able to secrete TssM (Fig. 6B). A previous study showed that *bsaZ* is essential for the function of the *B. mallei* T3SS$_{AP}$ (55). Taken together, the results indicate that the T3SS$_{AP}$ is not required for the secretion of TssM.

Four T6SS gene clusters—termed clusters 1, 2, 3, and 4—are present in *B. mallei* ATCC 23344 (46). The cluster 1 T6SS is located immediately upstream of *tssM* and is transcriptionally coregulated with *tssM* (46). To determine whether the cluster 1 T6SS was required for TssM secretion, we performed an immunoblot on the proteins present in the culture supernatants of five T6SS mutants using the polyclonal anti-rTssM antisera (Fig. 6C). A previous study demonstrated that the Δ*tssB*, Δ*hcp1*, Δ*tssD*, and Δ*tssE* mutants cannot secrete Hcp1, a putative effector molecule of the cluster 1 T6SS (46). Figure 6C shows that secretion of TssM by wild-type *B. mallei* was *virAG* dependent and that all five of the T6SS mutants were able to secrete TssM into the supernatant. The results demonstrated that the cluster 1 T6SS is not required for the export of TssM.

We next attempted to complement the mutation in the Δ*tssM* strain (DDA0729-1) by providing the *tssM* gene in *trans* on a broad-host-range plasmid. The Δ*tssM*::GFP mutation in DDA0729-1 was constructed by removing an internal fragment of *tssM* and replacing it with a promoter-less green fluorescent protein (GFP) gene (see Fig. S1 in the supplemental material). As expected, this strain did not exhibit green fluorescence in vitro unless *virAG* was supplied in *trans* on pBHR2 (see Fig. S1 in the supplemental material). The *tssM* gene was cloned into pBHR2 such that transcription was driven by the constitutive Cm promoter. As shown in Fig. 6B, we were able to complement the Δ*tssM*::GFP mutation in DDA0729-1 by providing pBHR2-*tssM* in *trans* (Δ*tssM*/*tssM*$^+$). The fact that *virAG* overexpression was not required for secretion of TssM in this strain further supports the finding the cluster 1 T6SS is not required for TssM export. In addition, this result confirms that the ATG at nucleotide position 556 is the correct *tssM* start codon (Fig. 1B).

***B. mallei tssM* is expressed shortly after uptake into macrophage cells.** Because ubiquitination of proteins occurs exclusively within eukaryotic cells, we hypothesized that TssM was produced by *B. mallei* inside eukaryotic cells to exploit Ub signaling and/or proteolysis pathways. To determine whether the *tssM* gene was expressed inside mouse RAW 264.7 macrophage cells, we used a strain of *B. mallei* in which *tssM* was fused to a promoterless GFP gene (see Fig. S1 in the supplemental material). In preliminary experiments, we observed that *tssM* was not expressed when *B. mallei* DDA0729-1 (Δ*tssM*::GFP) was incubated in DMEM alone (data not

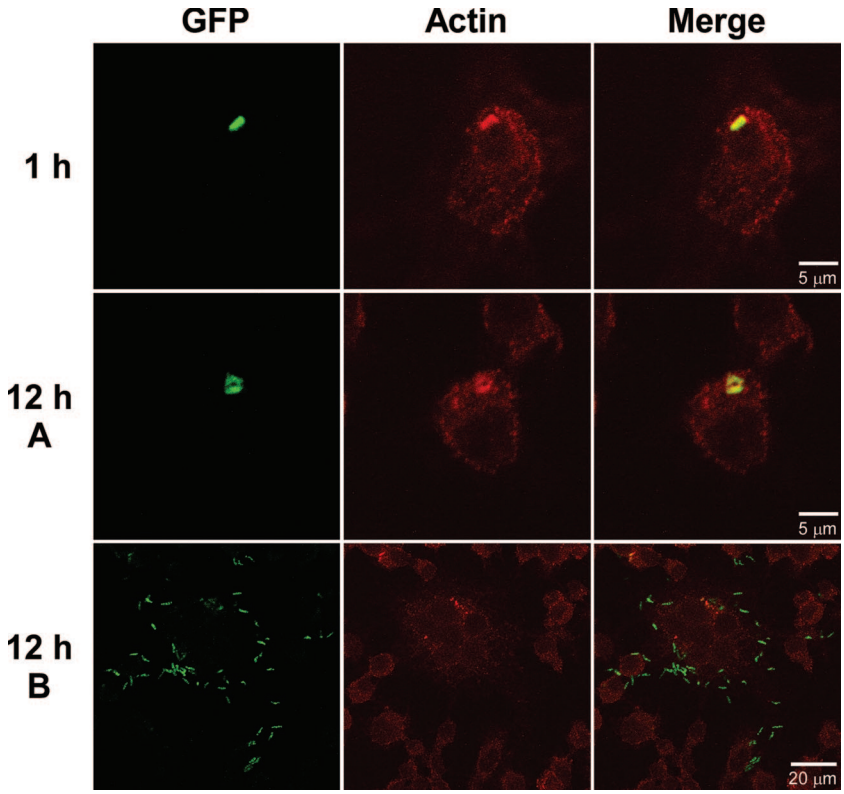

FIG. 7. Confocal micrographs of RAW 264.7 murine macrophages infected with *B. mallei* DDA0729-1 (Δ*tssM*::GFP). Fixed monolayers infected at an MOI of 40 were examined at 1 h and 12 h postinfection. Bacteria expressing GFP are shown in green and actin is shown in red. Two separate fields, A and B, are shown for the 12-h time point. White scale bars represent 5 or 20 μm as indicated. Micrographs are representative of at least two independent experiments.

shown). Macrophages were infected with DDA0729-1 at an MOI of 40 and were examined at 1 h and 12 h postinfection (Fig. 7). Within 1 h of infection, intracellular bacteria expressing GFP were observed (Fig. 7, 1 h). This demonstrates that *tssM* expression is responsive to a stimulus present within macrophage cells and that *tssM* is turned on relatively soon after *B. mallei* uptake. GFP-positive bacteria were also present at 12 h postinfection (Fig. 7, 12 h A), and bacteria that escaped RAW 264.7 cells into the extracellular milieu at this time point continued to express GFP (Fig. 7, 12 h B). The data clearly demonstrate that *tssM* is expressed inside RAW 264.7 murine macrophages within 1 h of infection. It is currently unknown whether *tssM* is expressed as a result of read through from the upstream T6SS promoter or whether a promoter immediately upstream of *tssM* is activated when *B. mallei* is inside RAW 264.7 murine macrophages.

***tssM* is not required for intracellular survival and replication inside RAW 264.7 macrophage cells.** We next compared SR1 (wild type) and DDA0729-1 (Δ*tssM*) for their ability to survive and replicate within RAW 264.7 murine macrophages at 3 h and 24 h (see Fig. S2 in the supplemental material). Both SR1 and DDA0729-1 survived and multiplied at an MOI of 1 but were killed at an MOI of 10, a finding consistent with previous studies (8). The survival of both strains was restored at an MOI of 10 when aminoguanidine, an inhibitor of inducible nitric oxide synthase (iNOS), was added to the cells (8) (see Fig. S2 in the supplemental material). Both strains induced multinucleated giant cell formation, a phenotype associated with maximal replication inside phagocytic cells in vitro (data not shown). The results showed that there was no quantitative difference between the ability of SR1 and DDA0729-1 to survive and replicate intracellularly, suggesting that TssM was not required for this phenotype in RAW 264.7 cells.

We next examined the cytokine profiles of RAW 264.7 cell culture supernatants after infection with SR1 and DDA0729-1 at an MOI of 10. There were no significant differences between these strains with regard to the kinetics or magnitude of tumor necrosis factor alpha, IL-1β, IL-6, and RANTES responses exhibited by RAW 264.7 cells at 6 and 24 h postinfection (data not shown). These results indicated that TssM is not involved in modulating the production of these cytokines in RAW 264.7 cells after infection with *B. mallei*.

Hamster virulence studies. Hamsters are highly sensitive to infection with virulent strains of *B. mallei* inoculated IP (18, 36). The LD_{50} for ATCC 23344 and SR1 (Table 1) in this acute model of glanders infection is <10 bacteria (46). DDA0729-1(pBHR2) and DDA0729-1(pBHR2-*tssM*) were examined for their relative virulence in the hamster model of infection. Groups of five animals were infected with 10, 100, and 1,000 CFU of each strain and monitored daily for 1 week. All animals in each group died within 5 days of infection, suggesting that the *tssM* gene is not required for virulence in this animal model of acute glanders infection.

DISCUSSION

Ubiquitination and deubiquitination regulate a broad range of processes in eukaryotic cells, including protein degradation, signaling, endocytosis, vesicular trafficking, cell cycle progression, and DNA repair (41, 53, 60). Recent reports suggest that several facultative intracellular bacterial pathogens exploit the host ubiquitin-proteosome system to their advantage by exporting DUBs or Ub ligases (3, 38, 45). We showed here that rTssM from *B. mallei* functions as a DUB that hydrolyzes Ub-AMC, K48-linked Ub, K63-linked Ub, and eukaryotic proteins conjugated to FLAG-tagged Ub. The k_{cat}/K_m value for rTssM hydrolysis of Ub-AMC was 10^7 M^{-1} s^{-1}, which was at least a thousandfold higher than those reported for other bacterial DUBs, including *Salmonella* SseL (10^2 M^{-1} s^{-1}) and *Yersinia* YopJ (10^4 M^{-1} s^{-1}) (30, 65). Note that *Yersinia* YopJ also functions as an acetylase, and its role as a DUB is somewhat controversial at the moment (37). In any case, the k_{cat}/K_m of rTssM was comparable to that of the widely studied eukaryotic DUB isopeptidase T (12). All bacterial DUBs described to date are members of cysteine peptidase clan CE (4, 45), but *B. mallei* TssM represents the first bacterial DUB in clan CA. This clan contains eukaryotic DUBs from cysteine peptidase families C12 and C19 and is defined by having the Cys box motif N-terminal to the His box motif (Fig. 1C) (4). In contrast, the His box motif precedes the Cys box motif in clan CE. The genus *Burkholderia* contains numerous species that interact with human, animal, or plant hosts (11), and it is tempting to speculate that this eukaryote-like DUB may have been acquired by a progenitor species via horizontal gene transfer. A BLASTP search (1) of the NCBI nr protein database identified TssM orthologs only in the closely related species *B. pseudomallei*, *B. thailandensis*, and *B. oklahomenesis*. The acquisition of this "eukaryotic" enzyme may have promoted the growth and survival of an ancestral *Burkholderia* species in its host(s), and it may have been positively selected for during the evolution of new species in this genus.

The mechanism of TssM translocation is currently unknown, but the results presented here demonstrate that the cluster 1 T6SS (46) and the animal pathogen-like T3SS (55) are not required. The fact that the T6SS did not play a role in TssM export was surprising given the fact that *tssM* is immediately downstream of the T6SS gene cluster and that both are positively regulated by the VirAG two-component regulatory system (46). The *B. mallei* genome encodes a variety of other secretion systems that may be utilized for TssM secretion, including a plant-like T3SS and three additional T6SSs (39, 61). The N terminus of TssM does not contain obvious Sec or Tat signal peptide sequences, and it may be secreted by a "nonclassical secretion" mechanism (6). SecretomeP 2.0 (www.cbs.dtu.dk/services/SecretomeP/) gives a 0.94 probability score to TssM, suggesting that it may be a nonclassically secreted protein. Further studies will be required to determine the exact mechanism of TssM secretion, but our immunoblotting results indicate that the secreted protein migrates as several distinct bands (Fig. 6). While the full-length ~50-kDa protein is present in both the cell pellet and the supernatant, several smaller TssM species are present exclusively in the supernatant. The nature of this putative processing event is currently unknown, but it is possible that the TssM protein undergoes autoproteolytic cleavage after secretion. Because the DUB active site is present at the C terminus of TssM, we predict that the N terminus of the protein is processed. The fact that we did not see any processing of rTssM supports this idea. We are currently trying to address this issue by constructing a chromosomal point mutation in the *tssM* cysteine residue critical for catalysis and examining the secretion profile of this mutant by immunoblotting.

The physiological substrate(s) of TssM is currently unknown, and DUB substrate identification has been an outstanding problem for years (32, 62). We found in the present study that rTssM efficiently cleaved FLAG-tagged Ub from eukaryotic proteins without any noticeable specificity for the protein substrate or the molecular nature of Ub conjugation (Fig. 5). It is important to emphasize the fact that rTssM only contains the C-terminal catalytically active domain of TssM and does not contain the N-terminal 192 amino acids. The N-terminal domain of herpesvirus-associated ubiquitin-specific protease (HAUSP) interacts with ubiquitinated p53 and brings it into close contact with the catalytically active domain of HAUSP (24). The N terminus of TssM may also serve as a specificity determinant, allowing the removal of Ub from the correct protein substrate(s) inside eukaryotic cells. Further studies will be required to identify potential ubiquitinated proteins with which the N terminus of TssM interacts.

Two-component regulatory systems are commonly used by pathogenic bacteria to adapt to different microenvironments during their infectious cycle (5). Previously, we demonstrated that overexpression of the *B. mallei* VirAG two-component regulatory system was required for the transcription of ~60 genes in vitro, including the T6SS gene cluster and *tssM* (46). Despite extensive testing, the physical and/or chemical environmental cues sensed by VirAG have not been identified (D. DeShazer, unpublished data). We hypothesize that VirA transduces a signal present within eukaryotic cells to VirG, which subsequently activates the transcription of genes that facilitate *B. mallei* growth and survival in this environment. In support of this notion, the genes required for *B. mallei* actin tail formation in the eukaryotic cytoplasm are upregulated by *virAG* (46). Recent experiments indicate that the *B. mallei* T6SS is required for optimal intracellular replication and multinuclear giant cell formation and it is likely that these *virAG*-regulated genes are also expressed in the intracellular environment (P. J. Brett and M. N. Burtnick, unpublished data). We demonstrated here that *tssM* was transcribed inside RAW 264.7 cells within 1 h of uptake, suggesting that TssM is produced early in infection and may play a beneficial role for *B. mallei* inside eukaryotic cells. Further studies will be required to identify the signal transduced by the VirAG two-component regulatory system, but it is likely to be found inside of host cells.

TssM was not required for replication or survival inside RAW 264.7 murine macrophages, and it had no effect on the production of various cytokines by these cells (see Fig. S2 in the supplemental material). It may be necessary to identify the physiological substrate of TssM before we can fully appreciate the role of this DUB in the bacterium-host cell interaction. Another possibility is that a murine macrophage-like cell line is not the optimal cell type for identifying a phenotype for the Δ*tssM* strain. *B. mallei* can invade epithelial cells in vitro (20), and this may be where TssM activity is most important. There

also was no difference in the relative virulence of wild-type and Δ*tssM* strains in the Syrian hamster model of glanders infection. While this result suggests that *tssM* is not a virulence factor of *B. mallei*, it is important to point out that hamsters are not the natural host of this pathogen. This animal model represents an acute model of infection (18), and it may be too sensitive to detect subtle virulence differences. It is possible that the only animals that will show virulence differences when infected with the wild-type and Δ*tssM* strains will be horses, mules, and donkeys. Given the massive gene loss and inactivation endured by this host-adapted pathogen during its evolution from *B. pseudomallei* (22, 39), it is unlikely that *tssM* has been maintained in the genome by chance. In fact, it would be surprising if it did not play at least a minor role during the interaction of *B. mallei* with solipeds.

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